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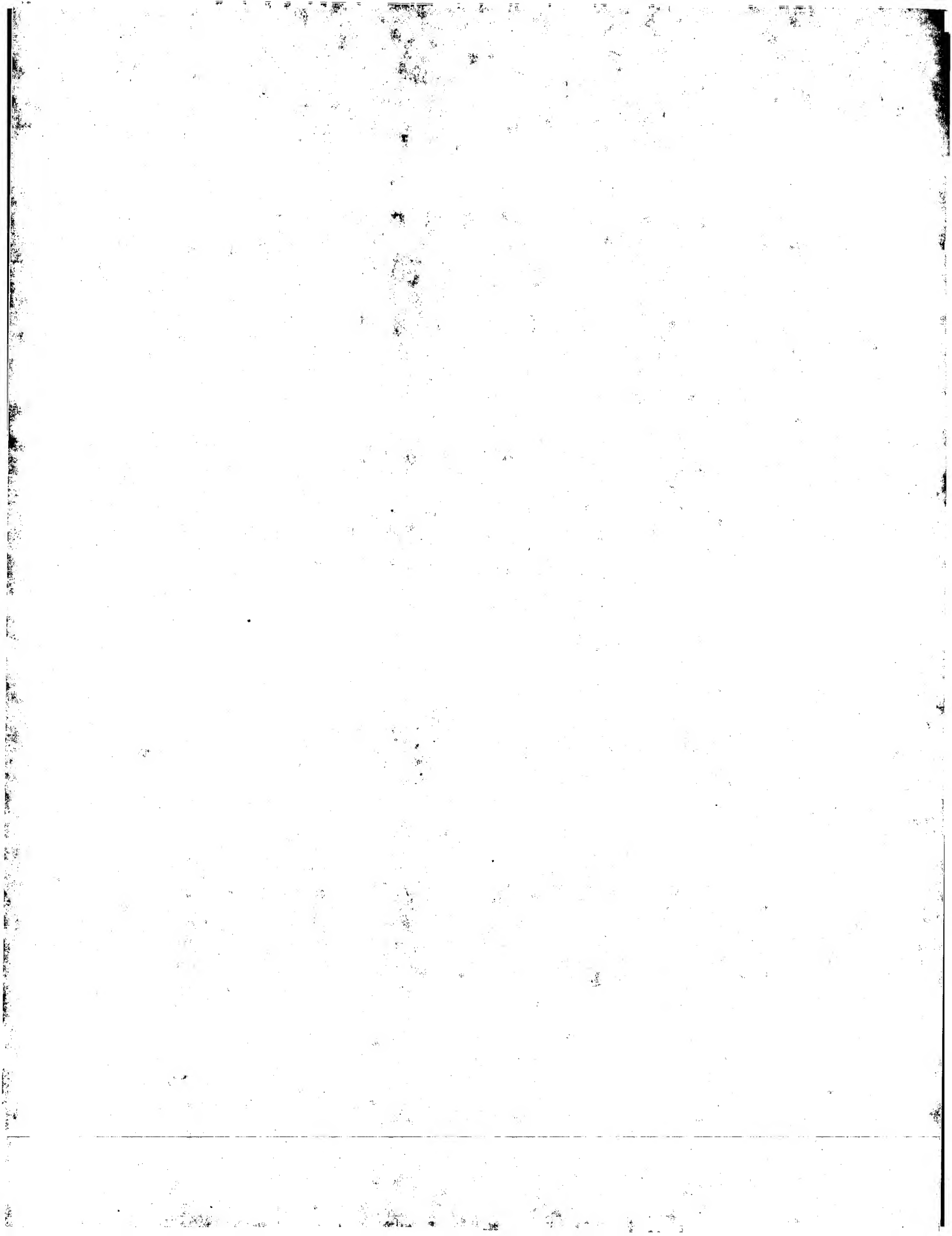
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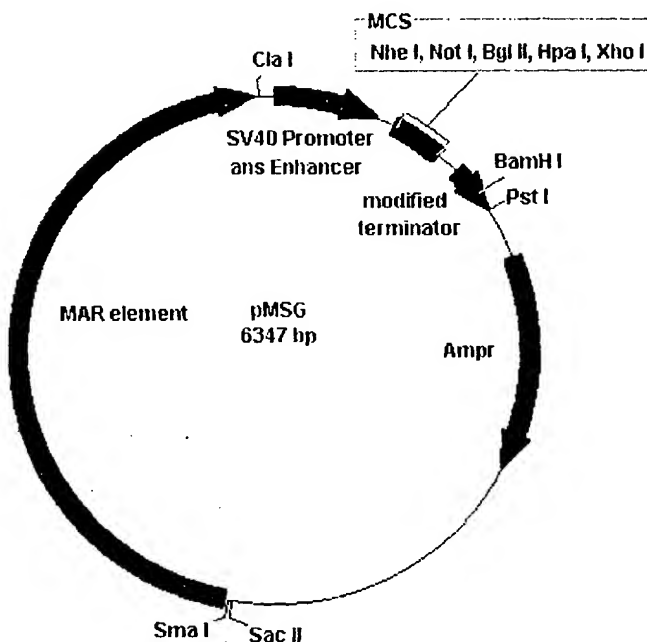
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[Continued on next page]

(54) Title: **EXPRESSION VECTOR USING FOR ANIMAL CELL**



(57) Abstract: The present invention relates to a expression vector for animal cells. Specifically, the present invention relates to a expression vector, pMS vector, pSG vector and pMSG, vector, including the human β -globin 5' MAR complementary sequence or/and the transcription termination site of gastrin gene. An expression system using the expression vector of the present invention can successfully produce recombinant proteins in various animals cells and recombinant protein having a unique structure and function.



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EXPRESSION VECTOR USING FOR ANIMAL CELL

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The present invention relates to an expression vector for animal cell,
5 and more particularly, to an expression vector including nuclear matrix
attachment region element (hereinafter referred to as "MAR element") and
transcription termination site of gastrin gene.

(b) Description of the Related Art

Many kinds of expression systems, such as microorganism, plant,
10 yeasts, insect cells, and animal cells are currently in use for medical treatment
by expression of the desired proteins in a large amount. Among the many
kinds of expression systems, microorganisms are most easily used as the
expression system, and many kinds of microorganism systems are studied and
utilized as the expression system.

15 However, use of the microorganism expression system is limited in
some respect. First, though genes are expressed in a microorganism, the
structure and characters of the expressed protein are unlike that of animal
protein, because the microorganism has a different mechanism for expressing
proteins and modification mechanism of protein such as glycosylation,
20 phosphorylation, and amidylation. Therefore, a recombinant protein produced
from microorganisms has nearly no modification, or it is limited in the production
of proteins of which the function is not much affected by the difference of the
modification and structure of the proteins. In addition, when the recombinant

proteins that are expressed by microorganisms are used, a cleaning process for the contamination of the microorganism or toxin is needed.

Though animal cells are suitable for an animal's protein expression, the expression system using animal cells is not commonly used, because the
5 expression system using animal cells create higher production costs due to a lower expression efficiency of recombinant protein compared to that of microorganisms and animal cells.

An animal cell for industry, which is currently in use for an expression system includes CHO (Chinese Hamster Ovary), BHK (Baby Hamster Kidney),
10 and myeloma, and expression vector is introduced to the animal cell and an desired foreign protein is produced, as in microorganism.

Therefore, gene expression systems are modified by various methods, because a small amount of the foreign genes are expressed in general. For example, a cell strain of the CHO is cultivated in medium containing
15 methotrexate (hereinafter referred to as "MTX") which is an inhibitor to DHFR (Dihydrofolate reductase), in order to obtain CHO strain which is alive depending on MTX concentration, and highly expresses protein due to the increase in the copy number of genes.

Generally, when the foreign genes are expressed in an animal cell, the
20 foreign gene is co-transfected with vector having selective marker and transformed cells are selected through cultivation in the selective medium for many hours. However, the frequency of achieving highly expressing cell clone is low. The low frequency of foreign gene expression is due to chromosomal insertion of the foreign gene in an animal system, unlike microorganism. In

addition, though the insertion process of the foreign gene is successful, the expression of the foreign genes cannot be expected, since the inserted site of each gene differs and the expression of the gene depends on the inserted site (Grindley et al., 1987. Trends Genet. 3, 16-22; Kucherlapati et al., 1984. Crit. Rev. Biochem. 16, 349-381; Palmiter et al., 1986. Annu. Rev. Genet. 20, 465-499). Therefore, though the foreign genes are stably integrated, it may be expressed in a small amount, because most of the gene expression in animal cells is inhibited by the neighboring nucleic acid (Eissenberg et al., 1991. Trends Genet. 7, 335-340; Palmiter et al., 1986. Annu Rev. Genet. 20, 465-499).

In order to protect the expression of the foreign gene from position effects, the possibility of using nuclear matrix elements in several systems have been reported. The exemplary nuclear element includes an insulator element, nuclear matrix attachment region (hereinafter referred to as "MAR"), and scaffold attachment region (hereinafter referred to as "SAR").

Kalos (Kalos et al., 1995 Mol. Cell. Biol. 15, 198-207) suggested that when apolipoprotein B MAR combined minimal promoter transgene construct, the foreign gene was stably introduced in the host chromosome, so that the expressed amount of the transcript increased by about 200 times. Similar to the aforementioned method, it was reported that chicken lysozyme A MAR and β -interferon SAR are capable of increasing the expression level of the foreign gene in a vertebrate cell regardless of the chromosomal insertion site (Eissenberg et al., 1991. Trends Genet. 7, 335-340; Klehr et al, 1991. Biochemistry 30, 1264-1270). However, it has not been verified that the MAR and SAR are capable of increasing protein production in CHO cell strain, or that

the MAR and SAR are suitable for common use.

When an animal cell gene is expressed, mRNA synthesis occurs from the promoter and stops at the termination site. The levels of the expressed proteins are often influenced by the efficiency of transcription termination as well as the stability of the synthesized mRNA.

The transcriptional termination site which is included in an expression vector controls polyadenylation, and has an influence in the mRNA stability. The termination site includes poly-A signal, cleavage site, termination site, and the polyadenylation signal is AATAAA and is well studied. However, the cleavage site where polyadenylation occurs, and the termination site where the gene transcription is completed by RNA polymerization enzyme II are not well known. In addition, though it is reported that GU/U-rich region except the three kinds of critical region controls the polyadenylation of the mRNA, the detail mechanism is not known.

Expression vectors which are commonly used in animal cells contain poly-A signal of SV40 virus and BGH (Bovine Growth Hormone), and it has not been suggested that the specific terminator that improves mRNA stability and the expression level is developed in order to use the expression vector of animal cells.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide an expression vector having increased expression efficiency and levels for foreign genes in the expression of foreign proteins used in an animal cell system.

In order to achieve these objects, this invention provides an expression

vector comprising MAR (Nuclear Matrix Attachment Region) element or its complementary sequence at 5'- terminal end of a promoter.

Also, this invention provides an expression vector for animal cells comprising a construct consisting of SV 40 virus poly-A (polyadenylation) signal and transcription termination site of gastrin gene, wherein the construct has a sequence of SEQ ID No. 3.

Also, this invention provides a pMSG KCCM 10202 vector of SEQ ID No. 8, comprising complementary sequence of the human β -globin 5' MAR (nuclear matrix attachment region), and the construct consisting of the SV 40 virus poly-A signal and the transcription termination site of gastrin gene.

Also, this invention provides a preparation method for bioactive materials by using an expression vector for animal cells comprising a β -globin MAR element, or the β -globin MAR complementary sequence at the end of promoter 5'-terminal, an a vector comprising SV40 virus poly-A (polyadenylation) signal of SEQ ID No. 3 and the transcription termination site of gastrin gene, and a pMSG vector.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a structure of an expression vector, which is prepared by combining SV40 promoter and β -gal gene for reporter of gene expression;

FIG. 2 shows an expression frequency of β -Gal induced by various MAR and SAR elements that are used in the present invention;

FIG. 3 shows an activity of expressed β -Gal, which indicates an influence on β -Gal expression by the MAR and SAR elements of the present

invention;

FIG. 4 shows various mutant constructs form of the MAR element based on β -globin MAR DNA sequence of the present invention;

FIG. 5 shows the expression frequency and expression amount of β -Gal proteins, after the transfection of a vector introduced with MAR elements
5 into CHO cells;

FIG. 6 shows the Southern and Northern blot for confirming β -Gal gene copy number (a: a control group, b: the present invention), RNA number (c: a control group, d: the present invention), and copy number of neo gene as a
10 selective marker (e: a control group, f: the present invention) in animal cells transformed with pMS- β -gal or pSV- β -gal vector as the control;

FIG. 7 is a graph showing the relation between the copy number of β -Gal gene and expression yield of β -Gal in animal cells transformed with the pMS- β -gal or pSV- β -gal vector as the control group;

FIG. 8 shows an expression titer of pMS- β -gal vector in various cell
15 lines;

FIG. 9 shows an expression amount of the foreign protein depending on MTX concentration in a cell transformed with pMS- β -gal vector or a control vector;

FIG. 10 shows an expression titer of the scu-PA, after the scu-PA
20 (single chain prourokinase) was inserted to pMS vector and the pMC vector, compared to that of the control;

FIG. 11 shows a construct including the transcription termination site of

gastrin gene and SV40 poly-A signal;

FIG. 12 shows an expression amount of β -Gal in expression vector comprising a construct including the transcription termination site of gastrin gene and the SV40 poly-A signal;

5 FIG. 13 shows the pMSG structure of the present invention;

FIG. 14 shows an expression of the TGF- β SRII in the pMSG vector confirmed by an antigen-antibody reaction; and

FIG. 15. shows an expression amount of the TGF- β SRII which is produced by cloning the TGF- β SRII gene to the pMSG vector, transfecting
10 them in a CHO cell, and culturing in condition of adding MTX.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

Hereinafter, the present invention will be explained in detail.

The inventors overcame problems arising from the site-specific effect when foreign genes are expressed in animal cell systems, and designed an
15 optimal expression vector that increases the expression amount of the genes.

An expression vector for animal cells of the present invention comprises suitable base sequences, which are further added to the conventional expression vectors. The suitable base sequences includes a nuclear matrix attachment region (hereinafter referred to as "MAR") and a scaffold attachment
20 region (hereinafter referred to as "SAR"), which stimulate the foreign gene expression of a host such as CHO (Chinese hamster ovary) and BHK (baby hamster kidney) from position effects of insertion site, and increase the expression amount of the foreign genes.

The MAR or SAR element is added to the end of promoter 5'-terminal and the efficiency of the expression vector of the present invention is analyzed. The chromosomal DNA is isolated from the cell, and then the chromosomal DNA is cloned in *E. coli* through PCR (polymerase chain reaction) and sub-cloning, so that the DNA of the MAR and SAR elements are obtained.

Preferably, the MAR or SAR element is selected from the group consisting of chicken lysozyme 5' MAR (Phi-Van, L. and Stratling, W. H., Biochemistry 35, 10735-10742 (1996), gene bank #: X98408), chicken α globin 5' MAR (Krevskii, V. A., Mikhailov, V. S. and Razin, S. V., Mol. Biol. 26, 672-678 (1992), gene bank #: X64113), a human β -globin 5' MAR (Yu, J., Bock, J. H., Slightom, J. L. and Villeponteau, B., Gene 139(2), 139-145 (1994), gene bank #: L22754), CHO DHFR intron MAR (Kas, E. and Chasin, L.A., J. Mol. Biol. 198(4), 677-692 (1987), gene bank #: X06654), a human HPRT intron MAR (Sykes, R. C., Lin, D., Hwang, S. J., Framson, P. E. and Chinault, A. C., Mol. Gen. Genet. 212, 301-309 (1988), gene bank #: X07690), a human CSP-B gene flanking SAR (Handson, R. D. and Ley, T.J., gene bank #: M62716), and a human interferon β -gene flanking SAR (Mielke, C., Kohwi, Y., Kohwi-Shigematsu, T. and Gode, J., Biochemistry 29, 7475-7485 (1990), gene bank #: M83137).

The MAR and SAR were integrated in an expression vector for animal cells, and the β -Gal expression of the vector was induced in order to confirm the relation between the MAR/SAR and expression titer.

The 5'-terminal end of pSV- β -gal promoter was connected to the

multicloning site (hereinafter referred to as "MCS") by in vitro PCR mutagenesis and then a recombination vector(version I and version II vector) was obtained, as shown in Fig. 1. The MAR or SAR was inserted in front of 5'-terminal end of the SV40 promoter of the recombination vector pSV- β -gal version I or II, a
5 test vector was prepared, and the test vector were transformed to CHO DG44. The transformed CHO DG 44 was selected on media containing G418 (neomycine), and the expression titer was measured by staining β -Gal (blue staining with IPTG and X-Gal). To measure the expression titer, the expression frequency, a number of the expression cells, and the amount of β -
10 Gal expression were measured.

FIG. 2 shows an expression frequency of β -Gal induced by various MAR and SAR elements that are used in the present invention. The pSV- β -gal vector showed about 20 to 30 % of β -Gal expression frequency, whereas the test vector comprising β -globin MAR, CSP-B SAR or interferon β SAR
15 increased the expression frequency in positive cell line, and more particularly, the test vector comprising β -globin MAR showed about 70 to 80 % of β -Gal expression frequency

In addition, constructs were prepared according to a combination of various MAR elements and SV40 promoter and influence of recombination
20 protein expression was compared to SV40 virus promoter. In order to compare the amount of β -Gal production, β -Gal staining method and measurement method of β -Gal enzyme were operated and the activity of β -Gal was analyzed in the same number of positive cell lines, since the expression frequency differs

according to each MAR element

Fig. 3 shows the activity of expressed β -Gal according to the influence of various MAR and SAR elements, and the β -Gal amount per positive cell or the activity of β -Gal increased, when the β -globin MAR, CSP-B SAR, and interferon β -SAR elements were used, compared to that of pSV- β -gal. It is observed that the expression amount of the vector comprising the β -globin MAR element increased by 7 times or more.

Accordingly, among MAR elements, the β -globin MAR element is more preferable in the present.

The DNA sequence of the MAR element was analyzed in order to investigate the effect and efficiency of the β -globin MAR element.

The β -globin MAR element includes 2,999 base and their function is not found in detail. The β -globin MAR element comprises a consensus sequence and 244 bp of alu element which is located at 3' of 800 bp region and, where A+T(Adenine, Thymidine) rich sequences exist. It is reported that the alu site comprises 300 bp of two directly repeating monomer units, and its recombination often occurs at this site, since thousands of homologous site exist in chromosome of an eukaryote (Jagadeeswaran et al., 1982. Nature 296, 469-470; Rogers. 1985. Int. Rev. Cytol. 93, 187-279). When the alu of β -globin MAR exists in upstream of SV 40 promoter, and the cell is cultivated for a long time, the recombination can occur.

Therefore, the inventors designed the β -globin MAR mutants that do

not have the aforementioned bad effect.

Fig. 4 shows the β -globin MAR mutants, which is produced by preparing the MAR complementary sequence b, deletion mutant c,d,e,f,g and i, and integrating them to the pSV- β -gal version I.

5 After the vector comprising the β -globin MAR mutant was introduced into the CHO cell, the cell number of β -Gal expression and the amount of β -Gal were measured, and the results are represented in Fig. 5. The β -Gal expression titer of most of the β -globin MAR mutants decreased; in contrast, that of the cell line transformed with pMS- β -gal vector comprising a β -globin
10 MAR complementary sequence was high. Figs. 6 and 7 shows the relation between expression amount of recombinant protein and the copy number of the integrated genes, and the expression amount of the pSV- β -Gal is independent on the copy number of the integrated genes, and the expression amount of the transformed vector with the pMS- β -Gal is proportional to the copy number of
15 the integrated genes. Since the β -globin MAR complementary sequence allows alu to exist on the other side of the promoter, the possibility of recombination of the vector decreases, and the expression amount increases by the prevention of the insertion site specific effect. Therefore, the β -globin MAR complementary sequence is preferable in the present invention.

20 It is verified that the MAR or SAR element is located at the 5' site of the conventional expression vector's promoter, and that the MAR or SAR mutants, or complementary sequence are integrated to the conventional expression

vector, so that the expression titer of the foreign proteins increase. Therefore, the present invention provides an expression vector including MAR or SAR element, and the expression vectors including MAR or SAR mutants, or their complementary sequence. The MAR or SAR is preferably selected from the group consisting of pi-a MAR (chicken pi α -globin 5'MAR), β -globin MAR (human β -globin 5' MAR), DHFR MAR (CHO DHFR intron MAR), HPRT MAR (human HPRT intron MAR), CSP-B SAR (human CSP-B gene flanking SAR element), interferon- β SAR element(human interferon- β gene flanking SAR element), and lyso MAR (chicken lysozyme 5' MAR).

Among them, pMS vector (KCCM 10203) of the present invention consisted of 6287 bp comprising the human β -globin MAR complementary sequence at 5' terminal end of a SV 40 virus promoter and multicloning sites, and are capable of expressing the recombinant protein by the integration of genes into the multicloning sites. The pMS base sequence is compiled as SEQ ID No. 1 with Sequence Listing software.

When the β -globin MAR complementary sequence is used, the expression frequency and the expression amount of the foreign genes increase by 3 to 4 times, and by 7 to 10 times, respectively, compared to that of the foreign genes when the only SV40 promoter is used. In addition, the pMS- β -gal vector is applicable to the various kinds of animal cell, which is represented in Fig. 8, and the pMS- β -gal vector system preferably applicable to BHK, CHO, NIH3T3, and HEK 293. Fig. 9 shows the expression titer increments by adding MTX (methotrexate) when the foreign proteins are expressed in the pMS- β -gal

vector system.

To measure the expression titer of foreign proteins according to MAR or SAR, CMV (cytomegalo virus) promoter is used. Since the expression titer of the foreign genes differs as depending on the kind of promoter and the various cell strains, the CMV promoter is also tested. In order to verify the effect of the β -globin MAR complementary sequence on the function of CMV promoter, pMC vector is prepared by a procedure similar to the pMS preparation, and scu-PA genes are integrated to pMS, pMC, and the control vector.

FIG. 10 shows that the expression titer of scu-PA in CHO transformed with pMSPUK, pMCPUK, pSPUK, and pMCPUK vectors which is prepared by integrating the scu-PA into the pMS, pMC, pSV, and pCMV, and that the gene expression of the pMS and pMC increased by 4 times that of pSV and pCMV. Therefore, the β -globin MAR complementary sequence of the present invention can be used for protein expression of animal cells by suitable combination with the desired promoters.

Since the vector of the present invention including the β -globin MAR complementary sequence is integrated with foreign genes as the host cell, useful proteins can be obtained from an animal cell strain in the conventional method.

In the present invention, a human gastrin gene transcription poly-A signal, a cleavage site and a human gastrin termination site were prepared, and they were applied to the expression vector of the present invention in order to increase the mRNA stability, thereby increasing the efficiency of the expression

vector.

The human gastrin gene, 3' transcriptional regulatory region comprises 605 bp including poly-A signal, cleavage sites, a termination site, and the base sequence of the human gastrin gene, 3' transcriptional regulatory region is filed
5 as SEQ ID No.2. The cleavage site is located 15 bp downstream from the poly-A signal, and the termination site is located 220 bp downstream from the poly-A signal. The transcription of gastrin is completed at the termination site and cleavage and polyadenylation of mRNA occur at the cleavage site.

The present inventor designed that the construct consisting of SV 40
10 poly-A and the transcription termination site of gastrin gene, which is capable of increasing the expression titer of the genes, is shown in Fig. 11.

Fig. 11 shows the constructs consisting of the transcription termination site of gastrin gene and SV 40 poly-A, and the constructs are preferably selected from pSV-SPA (SPA; the signal of SV 40 polyadenylation) in "a", pSV-SPA-GTF (GTF; transcription termination site of gastrin gene) in "b", pSV-SPA-GTR (GTR; GTF complementary sequence) in "c", pSV-GPA (GPA; gastrin
15 polyadenylation signal) in "d", pSV-GPA-GTF in "e", pSV-GPA-GTR in "f", pSV-GMPA (GMPA; GPA mutant) in "g", pSV-GMPA-GTF in "h", pSV-GMPA-GTR in "i". The base sequences of the SPA-GTF, SPA-GTR, SPA-GPA, SPA-GPMA are listed as SEQ ID No. 3, No.4, No. 5, and No.6, respectively. All the
20 constructs were respectively integrated into the pSV- β -gal, and further introduced to a COS-7 cell strain in order to measure the expression titer of β -Gal. The expression titer is the expression amount of β -Gal, and it is

represented in Fig. 12. The pSG vector comprising the construct consisting of SV 40 poly-A signal and the transcription termination site of gastrin gene has an effect in increasing the expression amount of the protein 4 times. Therefore, the termination site is preferably SPA-GTF (the SV 40 poly-A signal and the transcription termination site of gastrin gene) to increase the expression titer. In the present invention, the constructs are used as the termination site of the conventional expression vectors, so that the expression amount of the foreign proteins increases. The exemplary expression vector of the present invention includes pSG.

The pSG vector is an integrated vector with SPA-GTF at its termination site, and 3309 bp. The base sequence of the pSG vector is listed as SEQ ID No. 7. The pSG vector was inserted with the β -Gal in order to measure its expression titer, and the expression titer of the pSG is 4 times-larger than that of the pSV vector, which results from stabilizing mRNA by a construct consisting of the transcription termination site of gastrin gene and the SV 40 virus poly-A signal.

Therefore, the vector comprising SPA-GTF can express the foreign genes in an animal host in a conventional manner, and useful proteins such as a bioactive material can be obtained.

In addition, the present invention provides an expression vector including the two sites that are capable of increasing the expression titer of the foreign genes. The two sites are the transcriptional termination site of linking transcription termination site of gastrin gene with the SV 40 poly-A, and the β -globin MAR complementary sequence.

Fig. 13 shows the pMSG structure of the present invention, 6347 bp of total base sequences are listed as the SEQ ID No. 8, and pMSG was deposited under KCCM 10202 in Korea Culture Center of Microorganisms. The following Table 1 shows the map of the pMSG in detail.

Table 1

| SEQ ID No. | Function |
|------------|--|
| 1-419 | The early promoter of SV 40 and enhancer |
| 420-448 | Multicloning sites (MCS) |
| 449-656 | Transcriptional termination site |
| 3365-6329 | β -globin MAR complementary sequence |
| 1272-2132 | β -lactamase (AmpR) |

The protein for a TGF- β SRII gene, that is capable of preventing the side effects of the TGF- β by selectively-binding with the TGF- β that is an active human protein. The TGF- β SRII was expressed in order to analyze the effect and efficiency of the pMSG of the present invention. As a result, in Fig. 14, since the TGF- β SRII protein has a glycosylation structure, the protein has a greater molecular weight than that of the original protein, as typical animal cells have. The initial expression amount of the TGF- β SRII is about 100 ng/ 10^6 cells/day, and most of the cells are equally expressed. In addition, as in Fig. 15, $10\mu\text{g}/10^6$ cells/day were obtained at most, when 1 μM of MTX was added to the TGF- β SRII cells. The TGF- β has various functions in the human body, in particular, it is found as a factor resulting in inflammation such as corpora glomerulus sclerosis of kidney, hepatic cirrhosis, cornification of

epidermal cells, and an occlusal cartilage, the TGF- β SRII can be used for treatment of TGF- β -overexpressed disease.

The pMSG vector of the present invention has overcome a general problem, that is low expression yield and difficulty of obtaining transformants, and it is capable of mass production of the various kinds of recombinant proteins such as bioactive materials.

In addition, pMS vector (KCCM-10203) was developed, which is not inhibited its expression by neighbor base of insertion site, and pMS produced recombinant protein by about 8 times more than that of the conventional SV 40 promoter.

Also, the pSG vector was prepared, which comprises a transcriptional termination site that is capable of the induction of transcriptional termination at a specific site of transcripts and increasing by 3 times that of the conventional poly-A site.

The pMSG vector (KCCM-10202) of the present invention is prepared by the connection of the functional DNA fragments and multi-cloning site which is applicable to foreign genes, and the expression amount of it is $10 \mu\text{g}/10^6$ cells/day, when the TGF- β is expressed in animal cells. Accordingly, the expression vector of the present invention is suitable for the expression of the recombinant proteins, and according to the present invention, a protein derived from an eukaryote which is expressed in a prokaryote can be produced in animal cells as a recombinant protein having the same structure and function compared to wild type protein.

The following Examples further illustrate the present invention in detail but are not to be construed to limit the scope thereof.

Example 1: The preparation of pMS- β -gal vector

(1) Preparation of pMS- β -gal vector

5 A pMS- β -gal vector was prepared as follows.

- ① genome DNA isolation from a G-2 cell in order to obtain a base sequence of human β -globin MAR

A genomic DNA is isolated from a G-2 cell in a human host with use of Wizard Genomic DNA purification kit (a product by Promega Co.), and the
10 purification procedure followed the experimental procedure supplied by the production company.

② Perform of genomic PCR and Subcloning

In order to obtain a fragemt of human β -globin 5' MAR, the purified genomic DNA was used as a template, and the sense primer BML1 and the
15 antisense primer BMR 1 for the β -globin MAR were used in genomic polymerase chain reaction (PCR). The BML1 and BMR1 were listed as a SEQ ID No. 9 and a SEQ ID No.10, respectively. The PCR was performed with 32 cycles. Table 2 shows the cycle numbers.

Table 2

| | Experimental condition | Cycle numbers |
|---|--|---------------|
| 1 | 94 °C, 2 min. | 1 |
| 2 | 94 °C, 30 sec. → 60 °C, 45 sec. → 72 °C, 45 sec. | 2-31 |
| 3 | 72 °C, 10 min. | 32 |

After the PCR, the PCR product was inserted to pT7blue vector (Novagen Co.), and the pT7blue/ β -globin MAR vector was prepared. The pT7blue vector is a TA cloning vector which is capable of cloning a PCR product directly.

- 5 ③ Preparation of recombinant vector pSV- β -gal version I and version II containing multicloning site (MCS)

In order to insert β -globin MAR of pT7blue vector at the upstream of the promoter of the pSV- β -gal vector, the pSV- β -gal version I and the pSV- β -gal version II were prepared.

- 10 First, in order to prepare the pSV- β -gal version I, after the pSV- β -gal was treated with a Spe I and Hind III, and 443 bp of Spe I/Hind III fragment including SV 40 promoter was purified from agarose gel with a gene clean III kit(BIO 101 Co.). The fragment was ligated to the linearized pBluescript SK(+) (Stratagene Co.) by Spe I/Hind III digestion, and pBluescript/SV40 I promoter
15 vector was prepared.

- Then, the pBluescript/SV 40 I promoter vector was treated with Sca I and Hind III, a fragment including the SV 40 promoter was purified from the agarose gel as in the same manner of the aforementioned, and the fragment was ligated with the linearized pSV- β -gal vector by Sca I and Hind III digestion,
20 so that the version I vector was prepared.

In addition, in order to produce the pSV- β -gal version II vector, after the the pSV- β -gal was treated with EcoR I and Hind III, and the 420 bp of EcoR I/Hind III fragment including SV 40 promoter was purified from the agarose gel

as in the same manner of the aforementioned, and the fragment ligated with the linearized pBluescript SK(+) by EcoR I and Hind III digestion, so that the pBluescript/SV 40 II promoter vector was produced.

5 The pBluescript/SV 40 II promoter vector was treated with Sca I and Hind III in order to purify the fragment including the SV 40 promoter from the agarose gel as in the same manner of the aforementioned, and the fragment was ligated with the linearized pSV- β -gal vector by Sca I and Hind III digestion, so that the pSV- β -gal version II vector was prepared.

④ Preparation of a pMS- β -gal vector

10 The pT7blue/ β -globin MAR vector was treated with Spe I and Sma I and DNA fragment in the size of 3 kb including the β -globin MAR was purified from a agarose gel, and the fragment was ligated with the linearized recombinant pSV- β -gal version I by Spe I and Sma I, so that β -globin MAR was cloned. In order to confirm orientation of the β -globin MAR, restriction enzyme Hind III
15 which exists in pSV- β -gal version I and β -globin MAR was treated, and confirmed that the β -globin MAR was cloned in reverse orientation to the pSV- β -gal version I vector.

(2) Expression titer of the pMS- β -gal vector

20 2 μ g of the test vectors including the pMS- β -gal vector were co-transfected to CHO DG44 with pSV2neo vector using DOSPER (a product by Roche), and the pSV- β -gal vector as control vector was co-transfected in the same manner. The transfected CHO DG44 cell was cultivated in a selective

medium that was MEM- α medium including nucleosides supplemented 10% heat-inactivated FBS and 850 $\mu\text{g}/\text{ml}$ G418 sulfate (a product by Calbiochem Co.). Stably-transfected G418-resistant transfectants were generated after about 2 weeks, and twenty stable clones expressing β -Gal for control vector and pMS- β -gal vector in G418-resistant transfectants were isolated. 40 positive clones were analyzed by Southern and Northern blotting in order to measure the copy number of the β -Gal and the neo gene and the amount of the β -Gal RNA which is transcribed from pSV- β -gal and the pMS- β -gal.

FIG. 6 shows the Southern and Northern blot for confirming β -Gal gene copy number (a: a control group, b: the present invention), RNA number (c: a control group, d: the present invention), and copy number of neo gene as a selective marker (e: a control group, f: the present invention) in positive clones expressing β -Gal (hereinafter referred to as "positive clones") for pSV- β -gal or pMS- β -gal vector, and FIG. 7 is a graph showing relation between the copy number of β -Gal gene and expression yield of β -Gal in positive clones for the pSV- β -gal or pMS- β -gal vector. Since the copy number of the β -Gal gene in each positive clone was varied as shown in Fig. 6a and 6b, each positive clone was classified in two groups, where one has a high copy number of β -Gal gene, and the other has a low copy number of β -Gal gene, relatively, and the groups were analyzed with each other. In the group having a high copy number of the β -Gal gene, though control group had a high copy number of β -Gal gene as shown in "a", the RNA amount of the β -Gal gene in these clones was low

relative to a DNA copy number as shown in "c". However, the RNA amount in a positive clones for the pMS- β -gal of the present invention represented in "d" was high. In addition, in the group having a low copy number of the β -Gal gene, the copy number of the β -Gal and the RNA amount in positive clones for pMS- β -gal vector were much higher than those in positive clones for the control vector, as shown in Fig. 6. Fig. 7 shows the relation between the copy number and the expression amount of genes. In Fig. 7, the mean copy number and expression level of β -Gal gene in pMS- β -gal vector clones (●) correspond about 10 times comparing to those of control vector clones (○). In addition, in case of high-copy group of β -Gal gene, the expression of the β -gal gene in control vector clones is independent on the copy number of the gene, and the pMS- β -gal as shown in Fig. 7.

(3) Expression pattern of the pMS- β -gal vector in various cell lines

In the expression of the foreign genes in animal cells, various kinds of cells as well as the CHO are also used, and the expression amount of the foreign genes was various in the various kinds of cells. In the present invention, it is clarified that the expression titer and the expression frequency of the foreign gene in transfected CHO cell increased, when the pMS- β -gal vector in a CHO cell host is used. Therefore, it was tested whether the vector of the present invention is applicable to animal cells of which the origination and morphology are unlike that of the CHO cells.

After pSV- β -gal vector and the pMS- β -gal vector were respectively co-

transfected to a baby hamster kidney cell (BHK), a mouse fibroblast cell (NIH3T3), and a human embryonic kidney cell (HEK293) with a pSV2neo vector, and they were cultivated in media including G418 for about 14 days, the frequency of the positive cell expressing β -Gal and expression titer in stable
5 transfectants for each vector were measured in the same manner as in (2).

Fig. 8 shows the expression titer of the pMS- β -gal in the various cell lines. When the BHK is used for a host cell line, the gene expression is similar to that in a CHO cell line. When the NIH 3T3 is used for a host cell line, the effect on the increase of the expression amount was low. When the HEK 293
10 is used for a host cell, the effect and amount of expression was similar to those of the control. The frequency of the positive clones was similar to the aforementioned. Therefore, the effects of the expression vectors of the present invention are varied in the kind of cells, and in particular, they are useful for CHO, BHK, and NIH 3T3 cells which are generally used in the expression of
15 animal cells.

(4) Establishment of expression system of the pMS- β -gal vector.

In the conventional expression vector system, highly expressing clones should be selected through tedious processes to isolate as many as possible in pooled primary transfectants, and to cultivate these clones for a long time to
20 increase expression level of the foreign genes. In order to overcome these problems and maximize the expression level of the foreign genes, the DHFR/MTX amplification system was established on the expression system using the CHO DG44 cell line.

The pMS- β -gal vector was transfected to the DHFR-CHO DG44 (hereinafter referred to as "CHO DG44") cell line, transfectants for pMS- β -gal vector was adapted to MTX and then the expression amount of the proteins was measured. The pMS- β -gal vector and the control (the pSV- β -gal vector) were respectively co-transfected to CHO DG 44 having lacking of DHFR genes with pDCH1P vector having the DHFR genes. The DHFR-transfected cell strains were cultivated in selective media, MEM- α medium lacking of nucleosides supplemented 10% heat-inactivated dialyzed FBS. Stable DHFR⁺ transfectants were generated after about 2 weeks, and the positive clones expressing β -Gal in stable DHFR⁺ transfectants were isolated by β -Gal staining. For the β -Gal staining to screen positive clones expressing the β -Gal in DHFR⁺ transfectants, cells were fixed by incubating in PBS containing 2% formaldehyde, and 0.2% glutaraldehyde at 4°C for 10 minutes, washed twice with PBS, and was treated with X-Gal. When the β -Gal is expressed, the cell appears blue since blue products are generated due to the decomposition of the X-Gal by β -Gal protein. The selected clones were treated in multiple stepwise increments of MTX concentration such as 10 nM, 20 nM, 50 nM, 100 nM, 400 nM, and 1 μ M. It took clones about 2 to 3 weeks to adapt to each MTX concentration cultivated. The expression amount of the β -Gal during gene amplification by MTX adaptation was analyzed with the conventional ELISA.

Fig. 9 shows the expression amount of the recombinant proteins in cells

transfected with the pMS- β -gal and the control, respectively, and adapted 1 μ M MTX concentration. It has been reported that when the expression of the recombinant proteins was amplified by DHFR.MTX amplification system in the CHO cell line, the expression level was upto about 10 μ g/ 10^6 cells/day which corresponds to 2.5% of the total proteins (Kaufman, 1997, Methods Mol Biol 62, 287-300). In the present invention, it is observed that about 100 to 1000 of copy numbers are amplified by gradually increasing the MTX amount. The expression amount of the β -Gal in clones for pMS- β -gal vector of control was measured. The expression amount of the β -Gal in control clones is varied with each clone. Considered that 20 μ g/ 10^6 cells is valuable industrially in recombinant protein production in animal cells, 25% of control vector clones belong to that, and 88% of pMS- β -gal vector clones of the present invention produced the recombinant protein above 20 μ g/ 10^6 cells. Compared to the cell strains having maximal expression amount, the pMS- β -gal of the present invention has a large amount of expression protein, and large amounts of proteins can be produced by inserting a foreign gene to the pMS (KCCM-10203). Therefore, when the expression vector of the present invention is used for expression of foreign genes, it results in a high expression yield and efficiency.

Example 2: Preparation of pSPUK, pMSPUK, pCPUK, and pMCPUK

(1) Preparation of pCMV vector

A pCMV vector was prepared in order to make cloning of a CMV promoter and a SV40 promoter to scu-PA genes easy. The PCR for obtaining

the CMV promoter, MCS site, and a transcriptional termination site of a pcCDNA 3.1 (+) (a product by Invitrogen Co.) was performed. A PCR sense primer is CMVL1 (SEQ ID No. 11), and an antisense primer is PAR1 (SEQ ID No. 12). The CMVL1 primer has Sac II, Cla I, Nru I sites, and the PAR1 primer has BsmI site. After a 1.4 kb of PCR product was digested with the Sac II and Bsm I, and it was ligated with a linearized recombinant pSV- β -gal version I in order to prepare pCMV.

(2) Preparation of pSPUK, pMSPUK, pCPUK, and pMCPUK

In order to prepare a recombinant expression vector for scu-PA expression in an animal cell, scu-PA genes are obtained by PCR for the genome DNA separated from a CHO cell strain having scu-PA genes originated from a human TCL-598 cell strain as a template. A sense primer PKL1 is filed as SEQ ID No. 13, and an antisense primer PKR1 is filed as SEQ ID No. 14. The PKL1 primer has Hind III restriction site, and the PKR1 primer has Sma I restriction site.

About 1.3 kb of scu-PA PCR product produced from the primers (a DNA fragment having a base No. -6 to a base No. 1293 of a human scu-PA) was cut by the Sma I and Hind III, and it was inserted to a plasmid, pCMV which is cut by the Hind III and EcoR V in order to prepare a pCPUK expression vector.

In addition, the recombinant pSV- β -gal version I vector was treated with the Sma I and Hind III in order to separate a fragment having SV40 promoter from it. This fragment was inserted to and joined with a pCPUK of control which is linearized by Nru I and Hind III in order to prepare a pSPUK expression vector.

The pCPUK was treated with Sac II and Nru I in order to insert the MAR element into it. The linearized pCPUK was inserted to and joined with a β -globin MAR element which was prepared by treating the pMS- β -gal vector with Sma I and SacII, in order to prepare a pMCPUK vector.

5 The pSPUK vector was treated with Stu I and Sac II in order to insert a β -globin MAR element into it. A β -globin MAR element, which was produced by treating pMS- β -gal vector with Su I and Sac II, was inserted to the pSPUK vector, and pMSPUK vector was prepared.

10 In brief, the pSPUK vector construct was the form produced by inserting scu-PA gene to the recombinant vector, pSV- β -gal version I of which β -Gal gene was removed, and the β -globin MAR complementary sequence was inserted to the pSPUK vector in order to prepare pMSPUK vector. A pCPUK vector construct was the form produced by inserting scu-PA to the pCMV vector, and the β -globin MAR complementary sequence was inserted to the pCPUK
15 order to prepare a pMCPUK vector.

(3) Test for efficiency of the pSPUK, pMSPUK, pCPUK, and pMCPUK vectors

① transfection of a recombinant scu-PA genes to a cell, selection of transformants and amplification of the gene

20 Four kinds of recombinant expressing plasmid, that is pSPUK, pMSPUK, pCPUK, and pMCPUK, were respectively introduced to DHFR-CHO cell (CHO DG44) in order to obtain a cell line stable enough to express scu-PA. 2×10^5 of CHO cells were placed in a 6-well plate, and it was incubated in 5% CO₂

incubator at 37°C for 24 hours. 1 μ g of the plasmids of the pSPUK, pMSPUK, pCPUK and pMCPUK and 10 ng of DHFR minigene were respectively mixed in a ratio of 100:1, and the mixture was transfected into the CHO cell in the method of lipofectamine, (GiboBRL), or DOSPER (Loche). After 6 hours, a medium was replaced with the fresh culture medium, and the cells were further incubated for 48 hours. The cells were subcultured on the medium to the selective medium in a ratio of 10:1 for about 2 weeks or more, and the medium was exchanged per about four or five days in order to form a cell colony. The cell colony was cultivated separately or together.

② Activity measurement of scu-PA secreted to the culture solution for cells

The amidolytic activity of the culture medium of cells, which was measured by use of S-2444 as a substrate, and its result was compared to reference urokinase activity. 1x10⁶ cells were placed in a 6-well plate with 2 ml of the culture solution, and they were cultivated in a 5% CO₂ incubator at 37°C for 17 hours. In order to measure the activity of the supernatant, 50 μ l of the serially diluted supernatant was placed in a 96-well plate, and it was mixed with 30 μ l of buffer solution (50 mM Tris/HCl (pH 8.8), 80mM NaCl, 0.02% Twin 80). 10 μ l plasmin (0.5 U/ml) was further mixed to the mixture, and the mixture was reacted at 37°C for 20 minutes in order to activate the recombinant scu-PA. 10 μ l aprotinin (100 KIU/ml) was added to the mixture in order to inhibit the plasmin activity, and 100 μ l chromogenic substrate solution (a mixture of the buffer solution and 6 mM of S-2444) was further added to the mixture, and it

was reacted at 37°C for 1 hour. The activity and the concentration of the scu-PA in the culture medium were measured by absorbance (optical density) measurement at 405 nm of the resulting solution with a microplate reader, and the results were compared to that of urokinase as a reference.

5 Fig. 10 shows the expression titer of the scu-PA according to transfecting pMSPUK, pMCPUK, pSPUK and pCPUK to CHO cell. The expression level from pMS and pMC vectors increases by 4 times more than that from the pSV and pCMV as a control.

Example 3: Preparation of pSG- β -gal vector

10 (1) Preparation of transcription termination site of gastrin gene and the pSG- β -gal vector

 After the sense strand of the transcription termination site of gastrin genes of SEQ ID No. 15 was synthesized, and an antisense strand of SEQ ID No. 16 was synthesized, the two were annealed. The annealing fragment
15 were treated with BamH I and Pst I, and they were integrated to and joined with linearized pSV- β -gal vector (a vector produced by Promega Co.) by BamH I and Pst I digestion at 3' of SV40 p(A) terminator, so that the pSG- β -gal was prepared.

(2) Measurement of the pSG vector efficiency

20 The pSG- β -gal vector was co-transfected to Cos-7 cell with pSV2neo vector by the using DOSPER (Roche), in order to measure the expression titer of the β -Gal.

 Fig. 12 shows the expression titer of β -Gal in pSG- β -gal vector, and the

pSG- β -gal vector that comprises the construct consisting of the SV40 poly-A and the transcription termination site of gastrin gene produces β -Gal by 4 times more than that of the conventional vector comprising SV40 poly-A. Therefore, a large amount of recombinant proteins can be produced by the transfection of the foreign genes cloned in the pSG vector.

Example 4

(1) Preparation of the pMSG vector

As shown in Fig. 13, a vector containing with the β -globin MAR complementary sequence and SPA-GTF was prepared by the PCR. In order to prepare the pMSG vector, the pMS- β -gal and the pSG- β -gal were used as templates, the PCR was performed three times, and the PCR producer was treated with a specific restriction enzyme, and joined together.

① PCR of the β -globin MAR element

A sense primer ML1 (Sequence number 17) and an antisense primer MR1 (Sequence number 18) were used for the PCR. After the PCR product was treated with Sac II and Cla I, it was integrated with the linearized pMS- β -gal vector by Sac II and Cla I digestion, and the pMS- β -gal/sc vector was prepared.

② PCR for obtaining multicloning sites and transcriptional termination sites.

A sense primer TL1 (Sequence number 19) and an antisense primer TR1 (Sequence number 20) were used in PCR for the transcription termination site of gastrin gene of pSG vector. The PCR product was sub-cloned with a

pGEM-T (a product by Promega Co.), which is a sort of TA cloning vector which is capable of cloning a PCR product directly, so that pGEM-T/MCSp(A) was prepared.

③ PCR for obtaining SV40 promoter and multicloning site

5 A sense primer PL1 (a sequence number 21) and an antisense primer PR1 (a sequence number 22) were used in the PCR for the SV 40 promoter and the multi-cloning site of version 1 vector of Fig.1. The PCR product was treated with Apa I and Bgl II, and then it was integrated with the linearized pGEM-T/MCSp(A) vector by Apa I and Bgl II digestion, so that the pGEM-T/SVMCSp(A) vector was prepared.

④ Preparation of pMS vector and pMSG vector

PGEM-T/SVMCSp(A) of Example 4 was treated with Apa I and BamH I in order to purify a DNA fragment consisting of SV40 promoter, multicloning sites, and a SV40 termination site, and the purified fragment was integrated to the linearized pMS- β -gal/sc vector by Apa I and BamH I digestion of ① in Example 4, so that the pMS vector was prepared. The pSG vector was treated with BamH I and Sca I in order to separate 950 bp of DNA fragment having GTF base sequence, and it was joined with the linearized pMS vector by BamH I and Sca I digestion, so that a pMSG vector was prepared.

20 (2) Measurement of expression titer in pMSG vector

The PCR for TFG- β SRII (TGF- β soluble receptor II, glycosylation protein) gene was performed with a sense primer TRI (SEQ ID No.23) and an antisense primer TRR1 (SEQ ID No. 24) in order to verify the expression efficiency of pMSG vector in a CHO host cell line and industrial application of

the pMSG vector. The PCR product, amplified TGF- β SRII, was inserted into pMSG and pSV vectors at Nhe I and Xho I site, and each resultant vector was co-transfected into CHO DG44 cells with pDCH1P having DHFR genes. They were cultivated in selective media, where only cell strain having DHFR genes could be grown. Stable DHFR⁺ transfectants were generated after about 2 weeks, twenty DHFR⁺ clones were isolated, and these clones were analyzed by the western blot in order to measure the amount of TGF- β SRII and find its characteristics.

Fig. 14 shows the western blot that indicates TGF- β SRII expression, wherein "a" is the TGF- β SRII expressed from the pSV vector clones, and "b" is the TGF- β SRII expressed from the pMSG vector clones. In case of control vector clones, TGF- β SRII expression in one clone among 25 clones was detected. Whereas in 23 clones among 27 clones expression of TGF- β SRII was detected, further more many clones expressed TGF- β at a high levels. In addition, when TGF- β SRII was expressed by the pMSG expression vector, it has a glycosylation structure, so that its molecular weight increased as glycoprotein of a typical animal cell. The average expression level of these primary clones for pMSG vector is about 100 ng/10⁶ cells/day.

The stable primary clones for pMSG/TGF- β SRII vector of the present invention were adapted to DHFR/MTX amplification system by treatment in multiple stepwise increments of MTX amount such as 10 nM, 40 nM, 200 nM, and 1 μ M, in order to increase the expression amount of the TGF- β SRII.

FIG. 15 shows an expression amount of the TGF- β SRII which is produced by cloning the TGF- β SRII to the pMSG vector, transfecting them in a CHO cell, and adapting to 1 μ M MTX concentration. In the control vector clones, many clones are not adapted at each MTX concentration during the gene amplification process by MTX treatment, whereas the pMSG/TGF- β SRII
5 vector clones are well adapted, compared to the control vector clones. It is supposed that the MAR element of the pMSG expression vector increases the expression amount of the DHFR genes as well as foreign genes. As shown in Fig. 15, when adapted 1 μ M MTX concentration, many clones for pMSG vector
10 which is produced TGF- β in about $10\mu\text{g}/10^6$ cells/day were obtained.

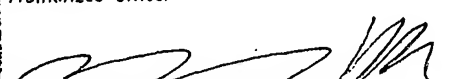
It has been reported that the TGF- β , a potent regulator of cell growth and differentiation, is central to the injury response. In a number of epithelia, repeated or prolonged injury leads to progressive fibrosis and ultimately the development of unwanted excessive scarring. In addition, TFG- β results in
15 disease such as corpora glomerulus, sclerosis of kidney, hepatic cirrhosis, cornification of epidermal cells, and an inflammatory cartilage. TFG- β SRII functions as antagonist of TFG- β . Therefore, the TGF- β is prohibited by the treatment of the TGF- β SRII as a medical treatment. The TGF- β SRII expressed from a CHO cell line of the present invention has an excellent
20 treatment effect compared to the proteins which are expressed from a prokaryote such as *E. coli*, or *Pichia pastoris*, and the expression vector of the present invention, which has an improved expression titer of the foreign genes,

may be used in animal cells.

INDICATIONS RELATING TO DEPOSITED MICROORGANISM
OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13bis)

| | |
|--|--------------------------------|
| A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page <u>16</u> , line <u>3</u> | |
| B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/> | |
| Name of depositary institution Korean Culture Center of Microorganisms | |
| Address of depositary institution (including postal code and country) 361-221, Yurim B/D, Hongje-1-dong, Seodaemun-gu, SEOUL 120-091 Republic of Korea | |
| Date of deposit 2000.7.28 | Accession Number KCCM 10202 |
| C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/> | |
| | |
| D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States) | |
| | |
| E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable) | |
| The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit") | |
| | |

| | |
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| <div>For receiving Office use only</div> <div><input type="checkbox"/> This sheet was received with the international application</div> <div>Authorized officer </div> | <div>For International Bureau use only</div> <div><input type="checkbox"/> This sheet was received by the International Bureau on:</div> <div>Authorized officer</div> |
|---|--|

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To: Mogam Biotechnology Research Institute

341, Pojung-ri Kosung-myun,
Yongin-city,
Kyonggi-do, 449-910
Korea (ROK)


RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

| | |
|--|---|
| I. IDENTIFICATION OF THE MICROORGANISM | |
| Identification reference given by the DEPOSITOR : <i>Escherichia coli</i> DH5 α /pMSG | Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM-10202 |
| II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION | |
| The microorganism identified under I above was accompanied by: <input type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable) | |
| III. RECEIPT AND ACCEPTANCE | |
| This International Depositary Authority accepts the microorganism identified under I above, which was received by it on Jul. 24. 2000 (date of the original deposit) ¹ | |
| IV. INTERNATIONAL DEPOSITARY AUTHORITY | |
| Name : Korean Culture Center of Microorganisms Address : 361-221, Yurim B/D Hongje-2-dong, Seodaemun-gu SEOUL 120-091 Republic of Korea | Signature(s) of person(s) having the power to represent the International Depositary Authority of of authorized official(s) : Date: Jul. 28. 2000. |

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired : where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

INDICATIONS RELATING TO DEPOSITED MICROORGANISM
OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13bis)

| | |
|---|---|
| A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page <u>12</u> , line <u>10</u> . | |
| B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/> | |
| Name of depositary institution Korean Culture Center of Microorganisms | |
| Address of depositary institution (including postal code and country) 361-221, Yurim B/D, Hongje-1-dong, Seodaemun-gu, SEOUL 120-091 Republic of Korea | |
| Date of deposit 2000.7.28 | Accession Number KCCM 10203 |
| C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/> | |
| | |
| D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States) | |
| | |
| E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable) | |
| The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g. "Accession Number of Deposit") | |
| | |
| <div style="border: 1px solid black; padding: 2px;">For receiving Office use only</div> <div style="border: 1px solid black; padding: 2px;"><input type="checkbox"/> This sheet was received with the international application</div> <div style="border: 1px solid black; padding: 2px;">Authorized officer </div> | <div style="border: 1px solid black; padding: 2px;">For International Bureau use only</div> <div style="border: 1px solid black; padding: 2px;"><input type="checkbox"/> This sheet was received by the International Bureau on:</div> <div style="border: 1px solid black; padding: 2px;">Authorized officer</div> |


BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To: Mogam Biotechnology Research Institute

341, Pojung-ri Koo-sung-myun,
Yongin-city,
Kyonggi-do, 449-910
Korea (ROK)

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

| | |
|--|---|
| I. IDENTIFICATION OF THE MICROORGANISM | |
| Identification reference given by the DEPOSITOR : <i>Escherichia coli</i> DH5 α /pMS | Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM-10203 |
| II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION | |
| The microorganism identified under I above was accompanied by: <input type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable) | |
| III. RECEIPT AND ACCEPTANCE | |
| This International Depositary Authority accepts the microorganism identified under I above, which was received by it on Jul. 24, 2000 (date of the original deposit) ¹ | |
| IV. INTERNATIONAL DEPOSITARY AUTHORITY | |
| Name : Korean Culture Center of Microorganisms Address : 361-221, Yurim B/D Hongje-1-dong, Seodaeun-gu SEOUL 120-091 Republic of Korea | Signature(s) of person(s) having the power to represent the International Depositary Authority of of authorized official(s): Date: Jul. 28, 2000 <div style="text-align: right;">  </div> |

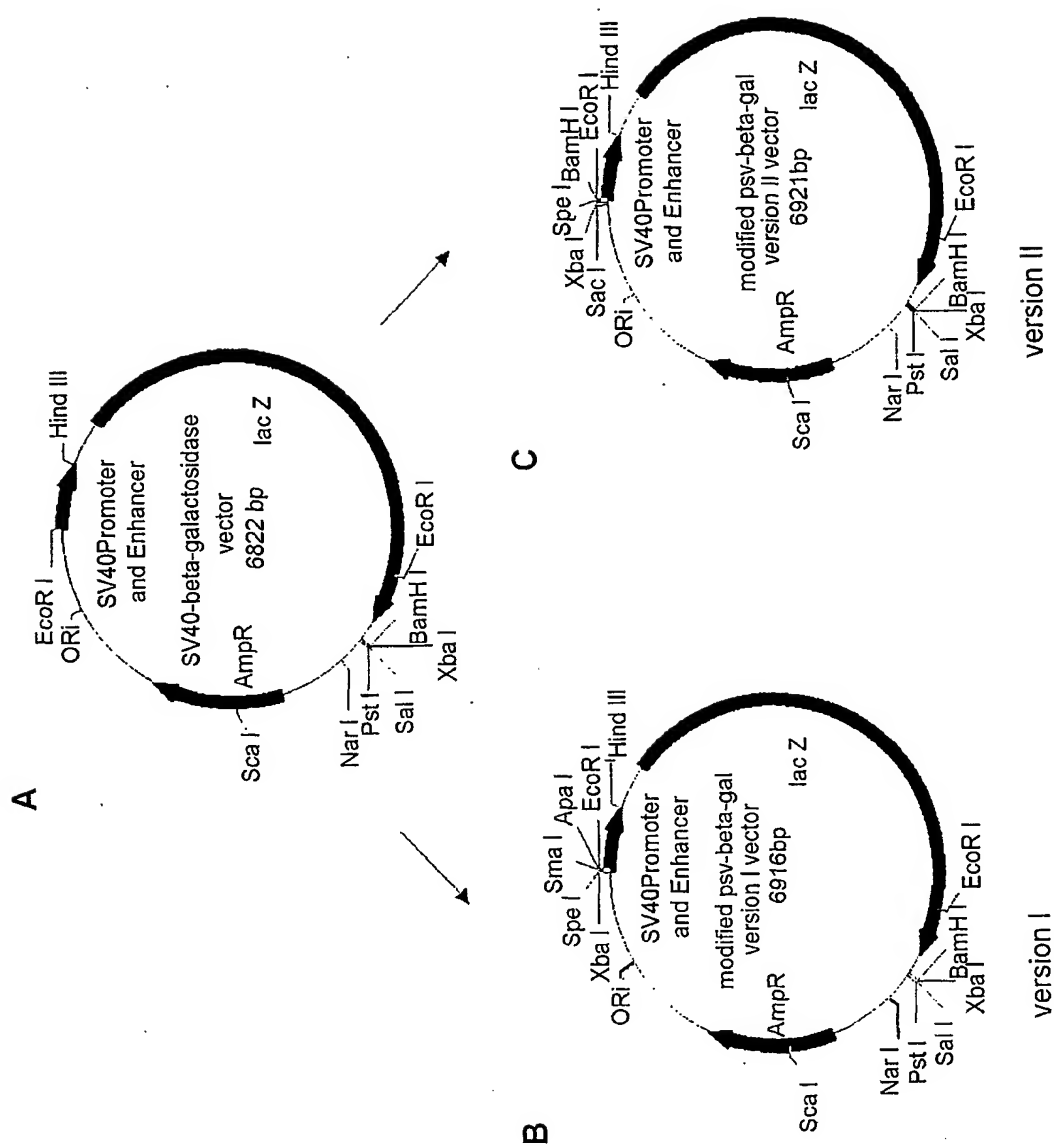
¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired; where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

WHAT IS CLAIMED IS:

1. An expression vector for animal cell comprising β -globin MAR sequence or its complementary sequence at 5'-terminal end of a promoter.
2. The expression vector according to claim 1, wherein the
5 expression vector for animal cell is pMS KCCM 10203 of SEQ ID No.1.
3. The expression vector according to claim 1, wherein the expression vector for animal cell is pMC.
4. An expression vector for animal cell comprising a construct consisting of SV 40 virus poly-A (polyadenylation) signal and transcription
10 termination site of gastrin gene, wherein the construct has a sequence of SEQ ID No. 3.
5. The expression vector according to claim 4, wherein the expression vector is pSG of SEQ ID No. 7.
6. A pMSG KCCM 10202 vector of SEQ ID No. 8, comprising the
15 human β -globin 5' MAR (nuclear matrix attachment region) complementary sequence, and the construct consisting of the SV 40 virus poly-A signal and the transcription termination site of gastrin gene.
7. A preparation method of bioactive material in animal cells from the expression vector selected from the group consisting of the expression
20 vectors according to claim 1, claim 4, and claim 6.
8. The preparation method according to claim 7, wherein the bioactive material is TGF- β SRII prepared from the expression vector according to claim 6.

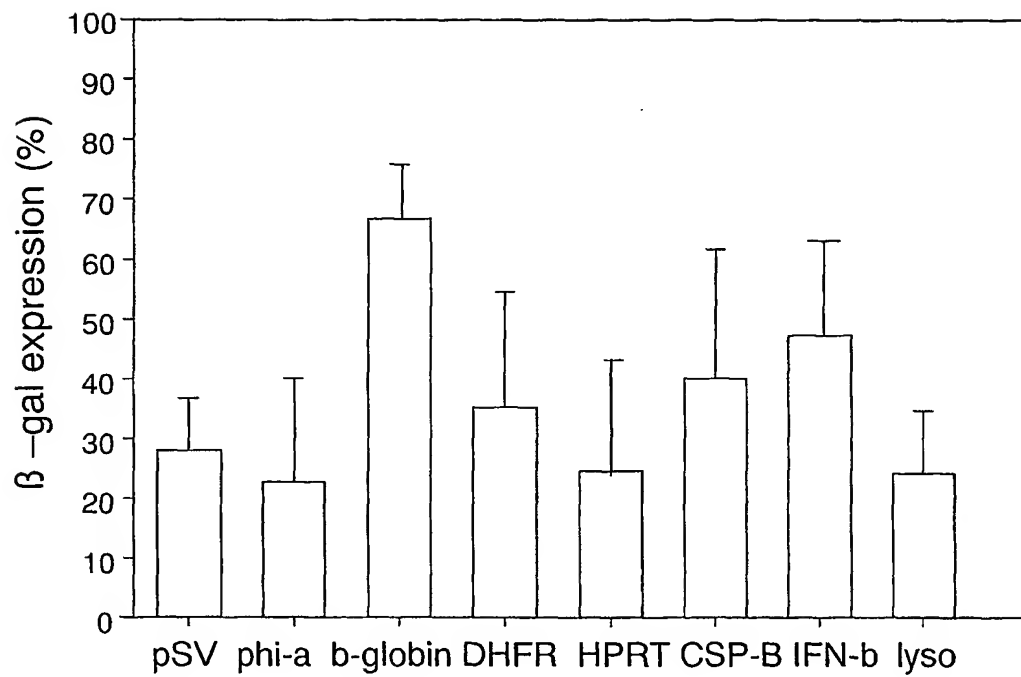
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FIG. 1



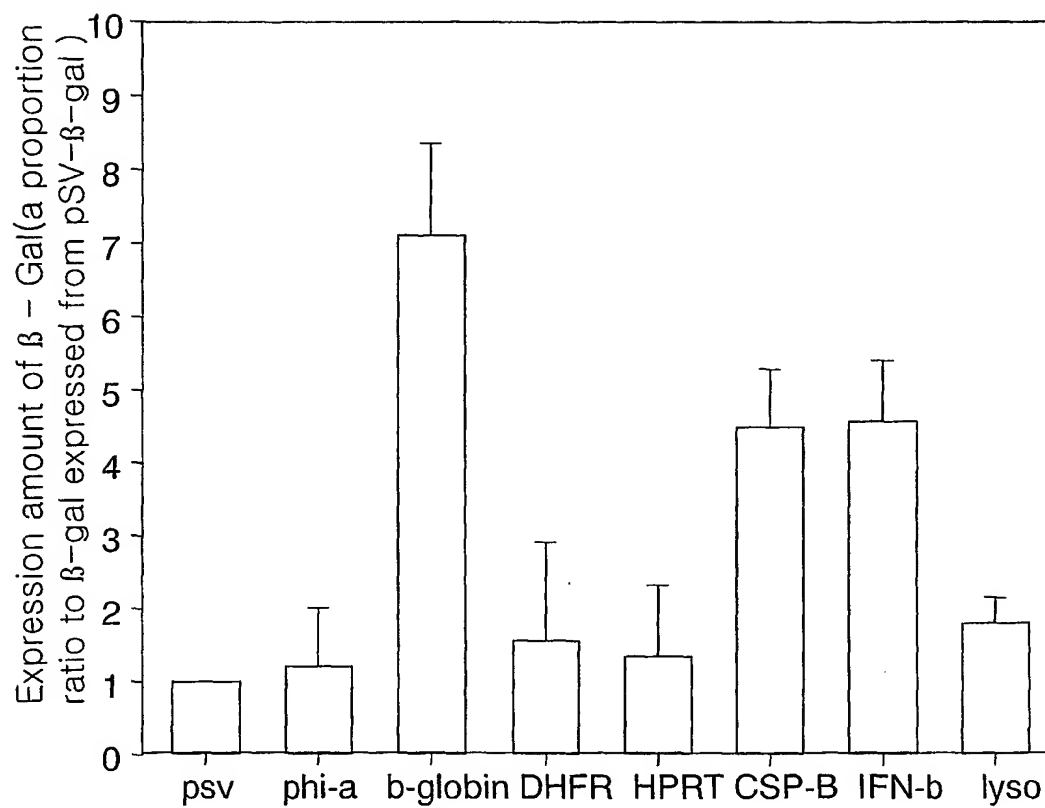
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FIG. 2



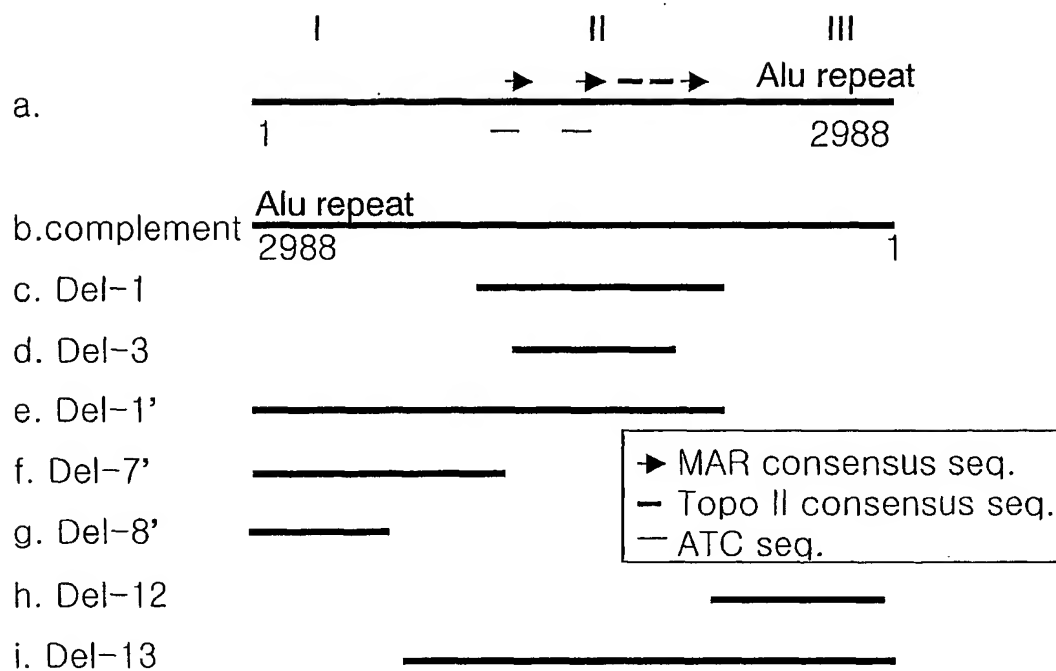
3/15

FIG. 3



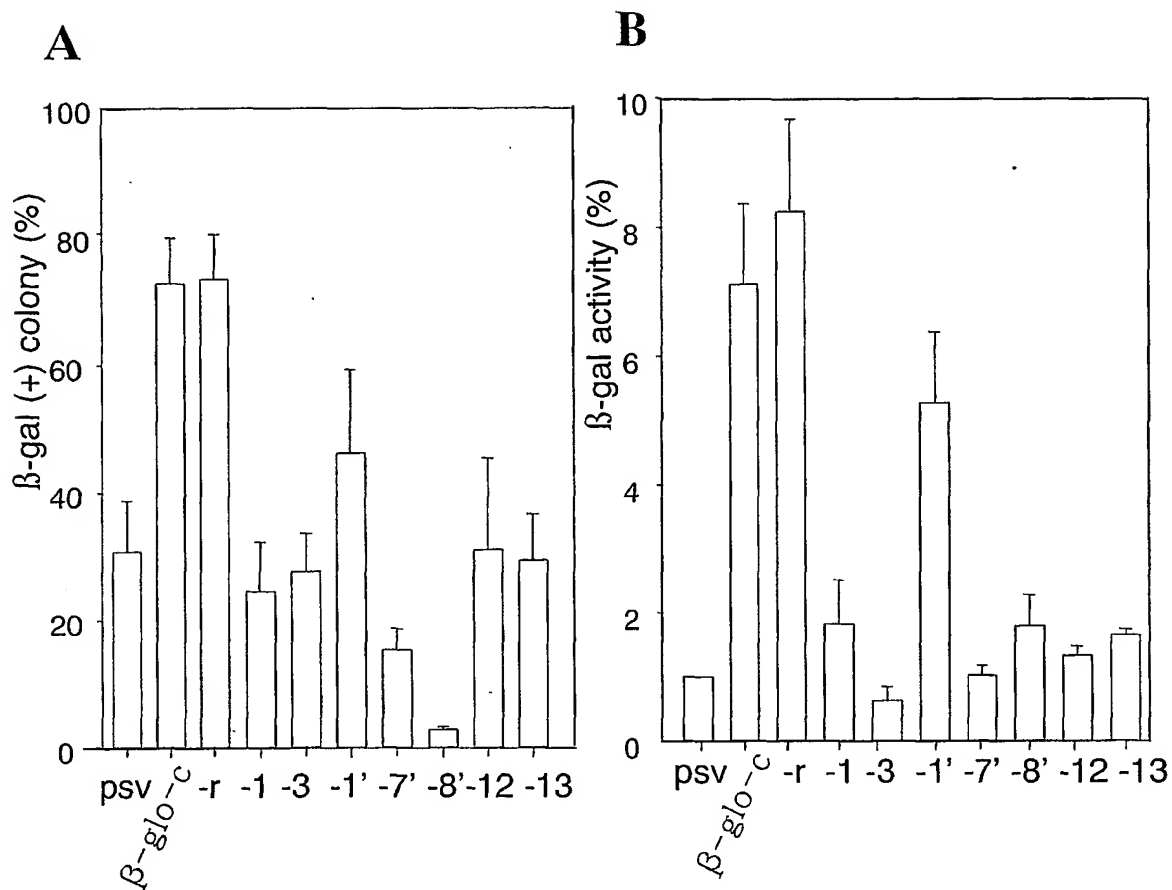
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FIG. 4



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FIG. 5

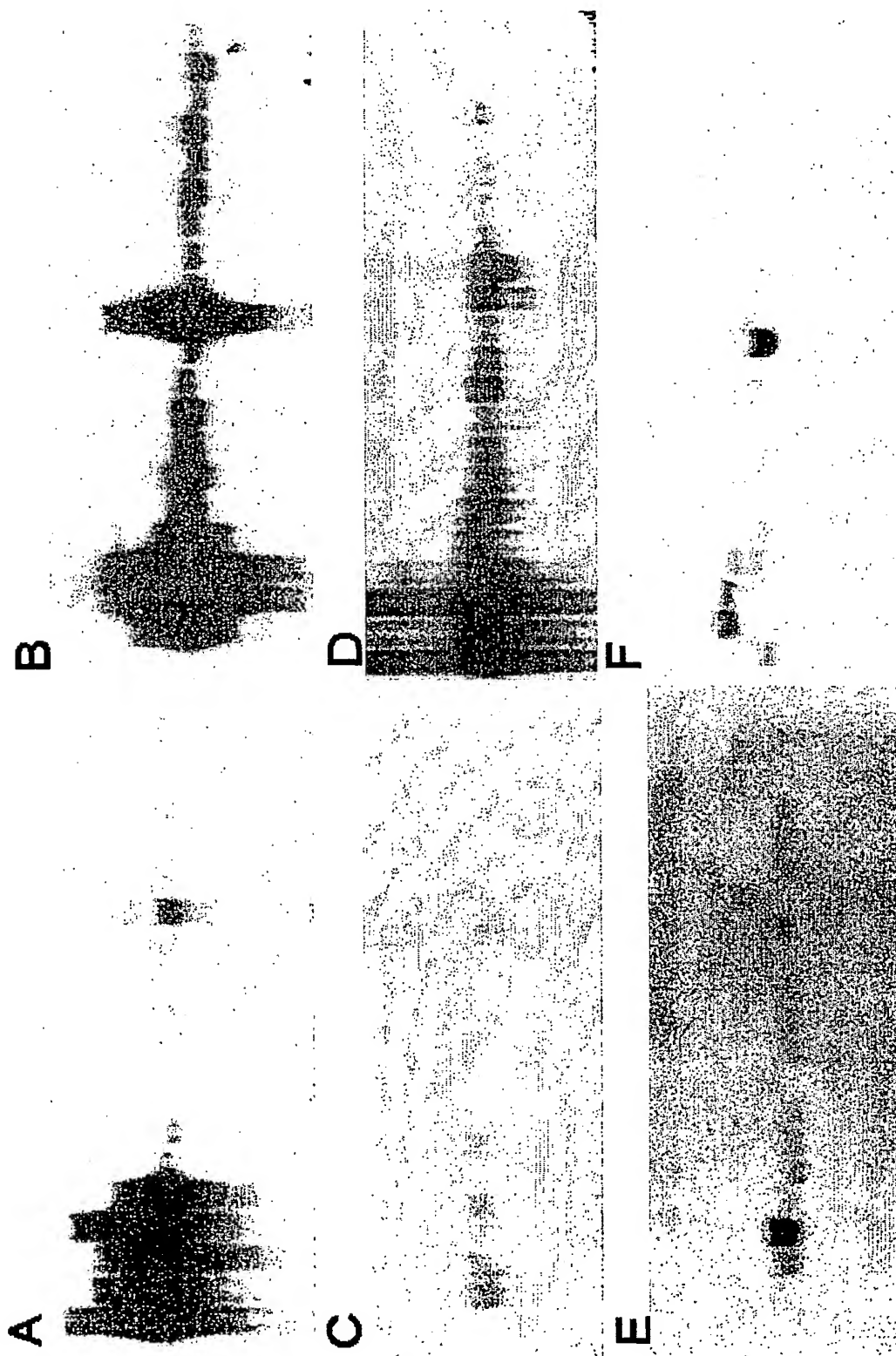


(β -glo-c : vector containing β -globin MAR,

β -glo -r : vector containing the β -globin MAR complement)

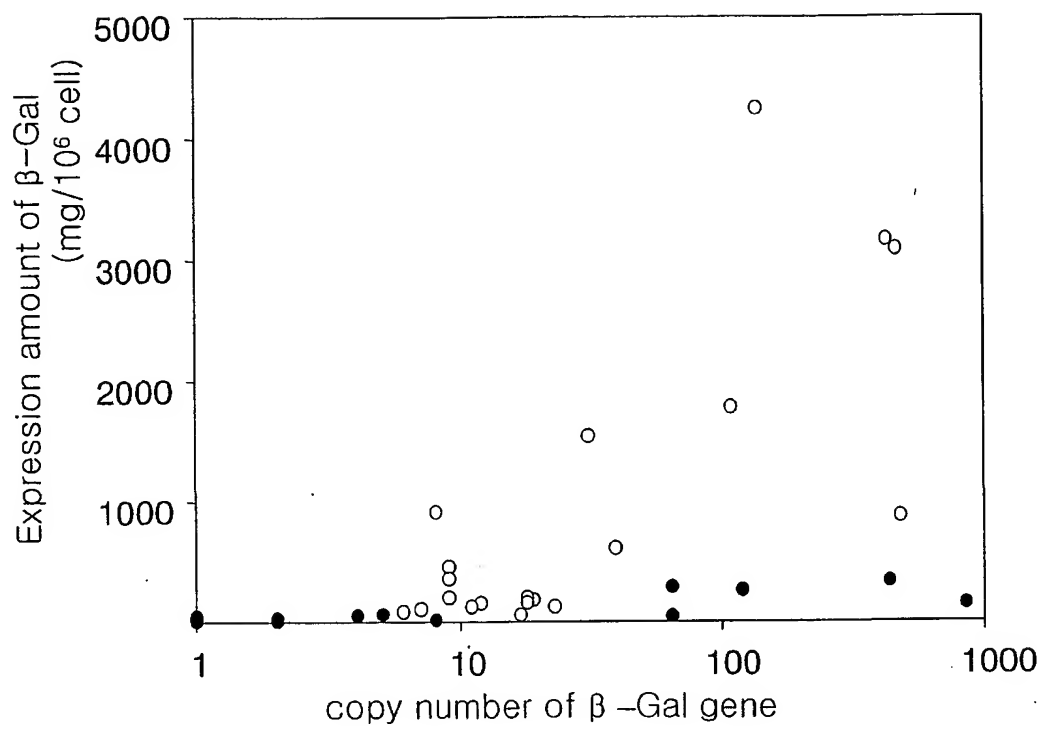
6/15

FIG.6



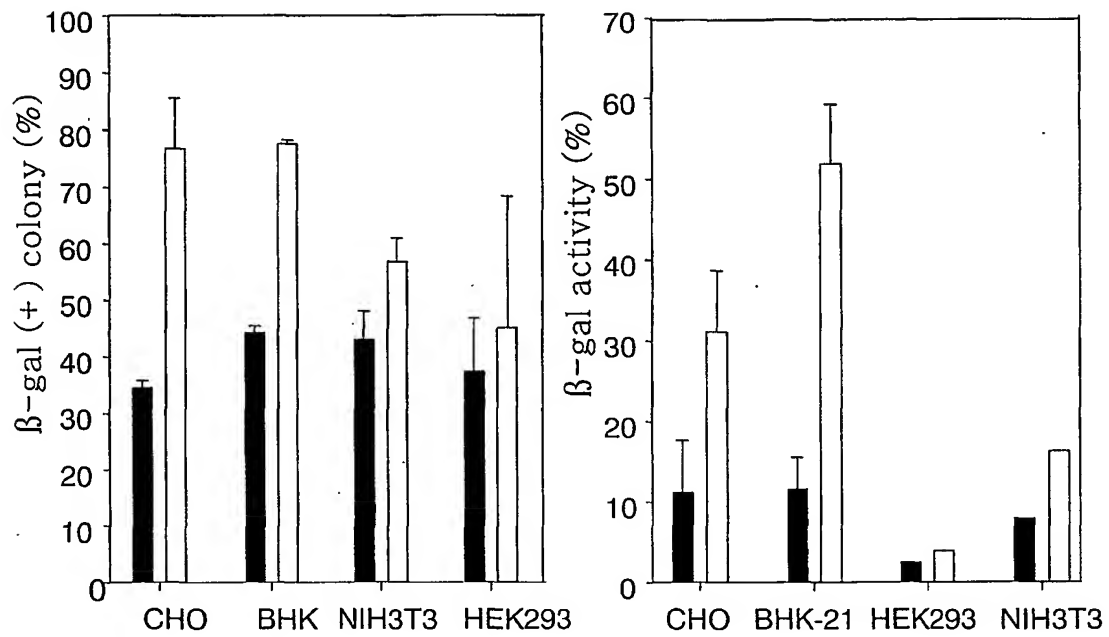
7/15


FIG. 7




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FIG. 8



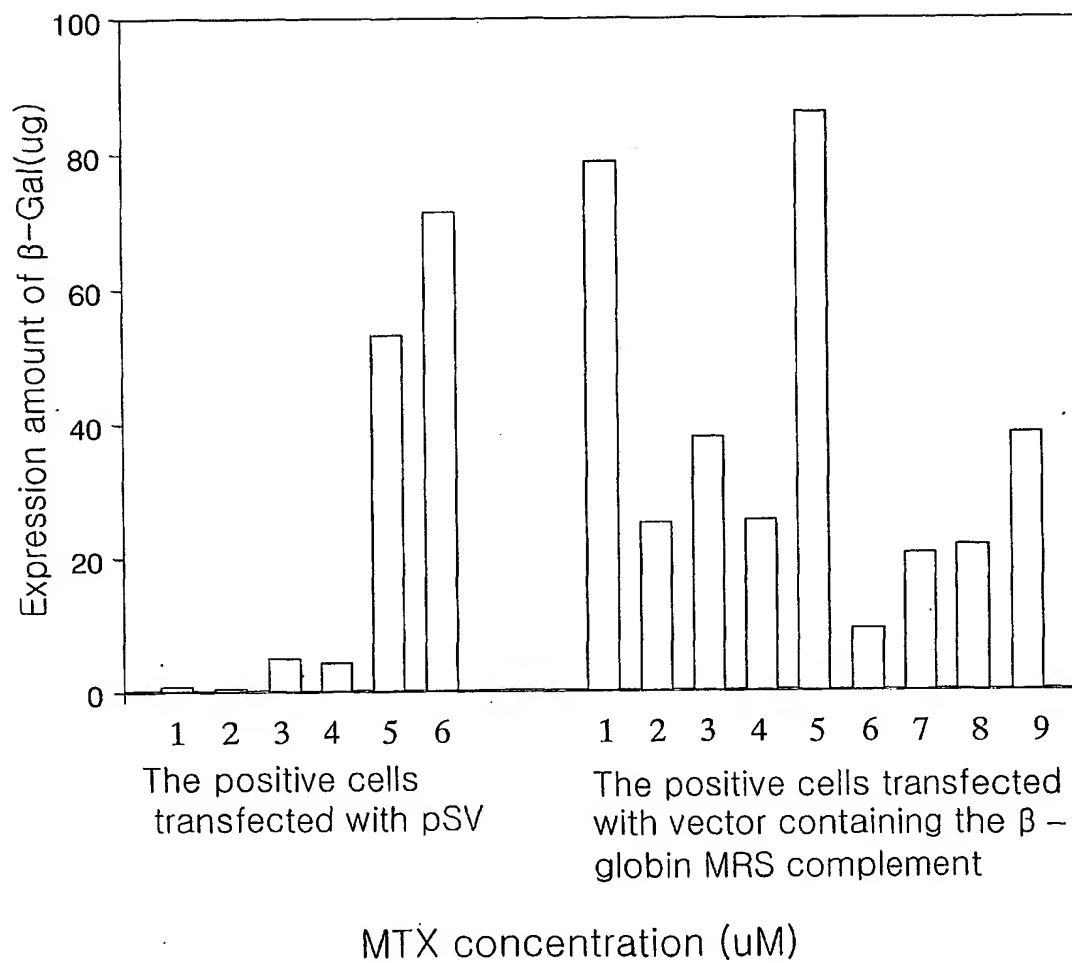
The positive cells transfected with control vector : 

The positive cells transfected

with vector containing the β -globin MAR complement : 

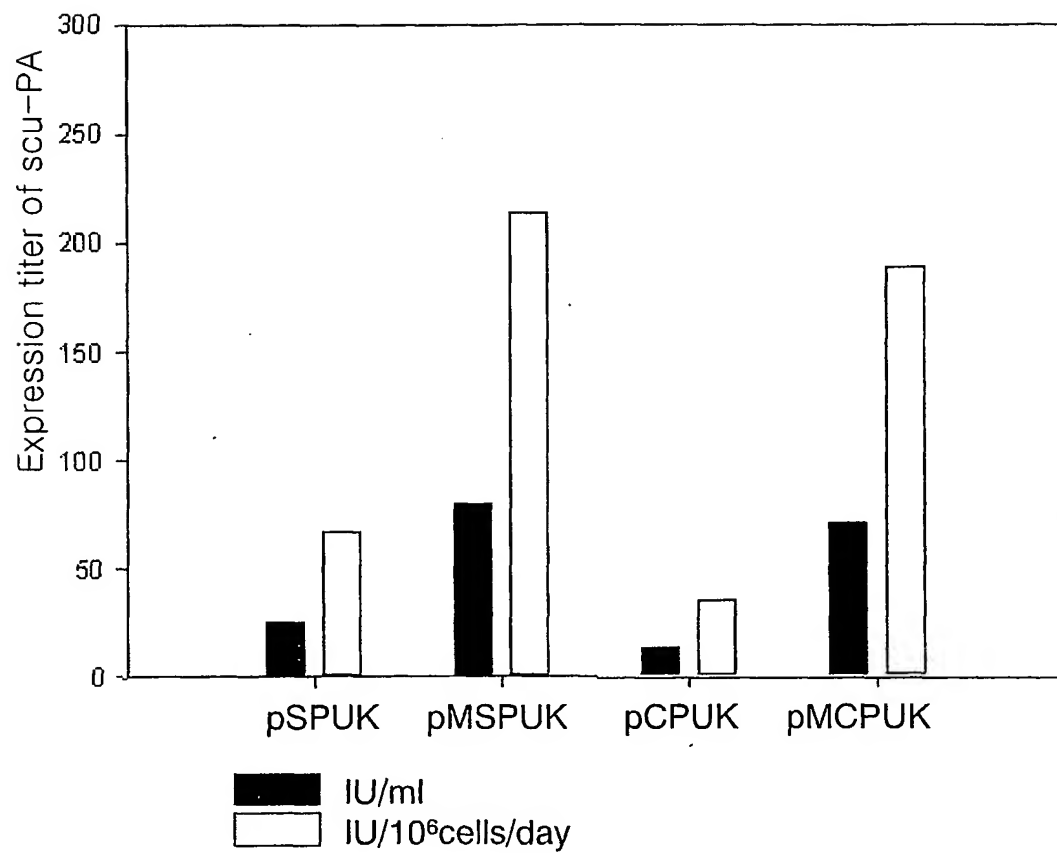
9/15

FIG. 9



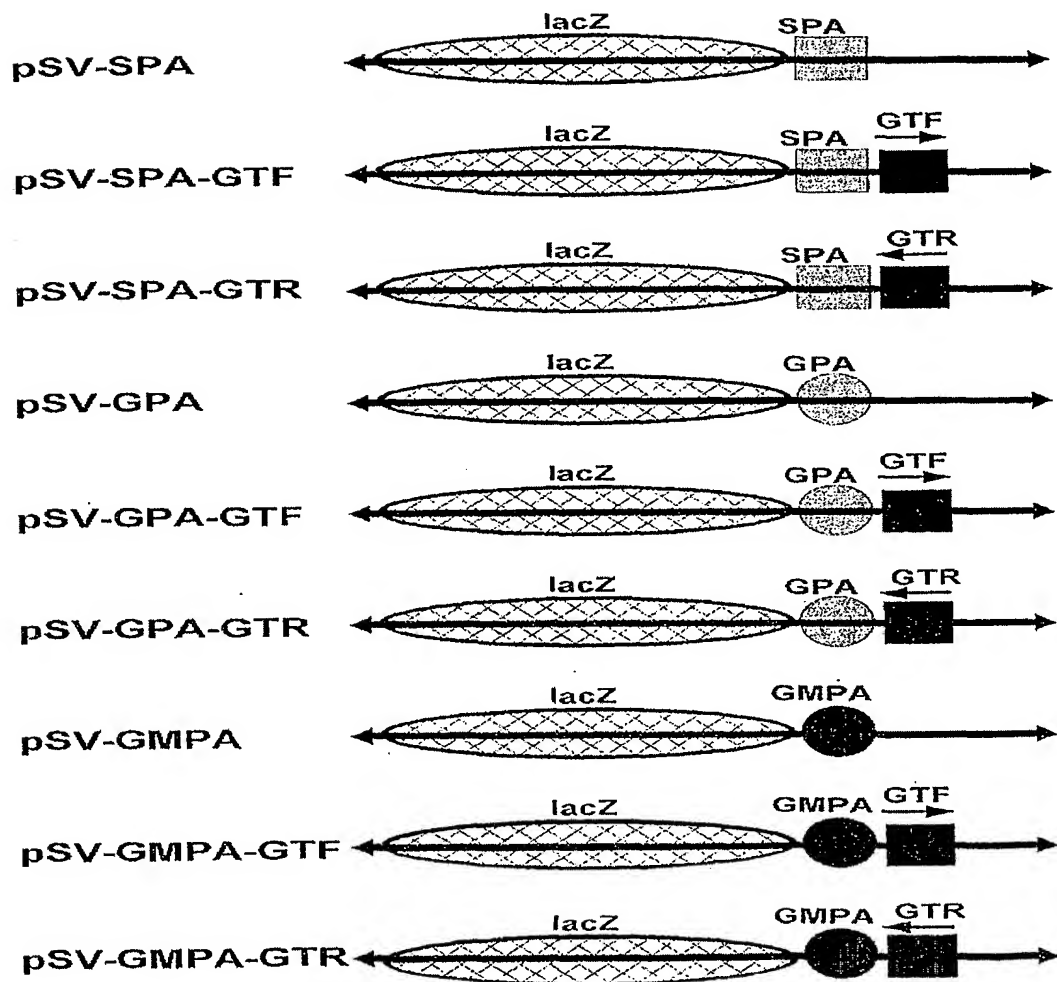
10/15

FIG. 10



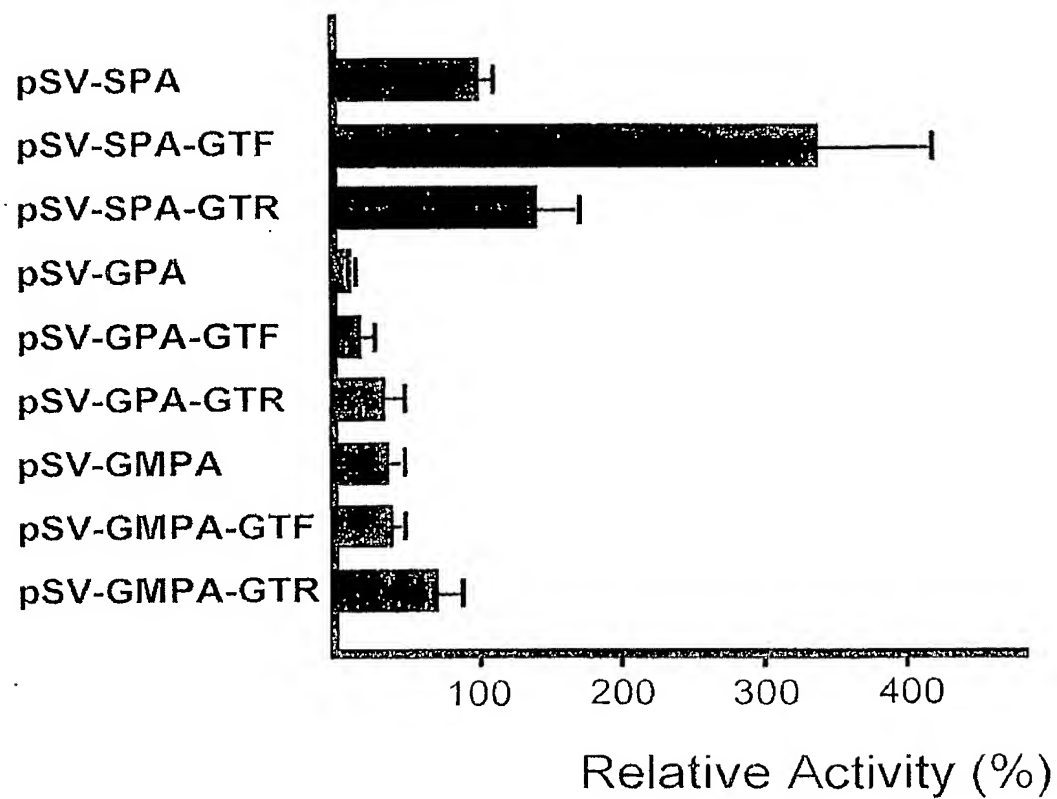
11/15

FIG. 11



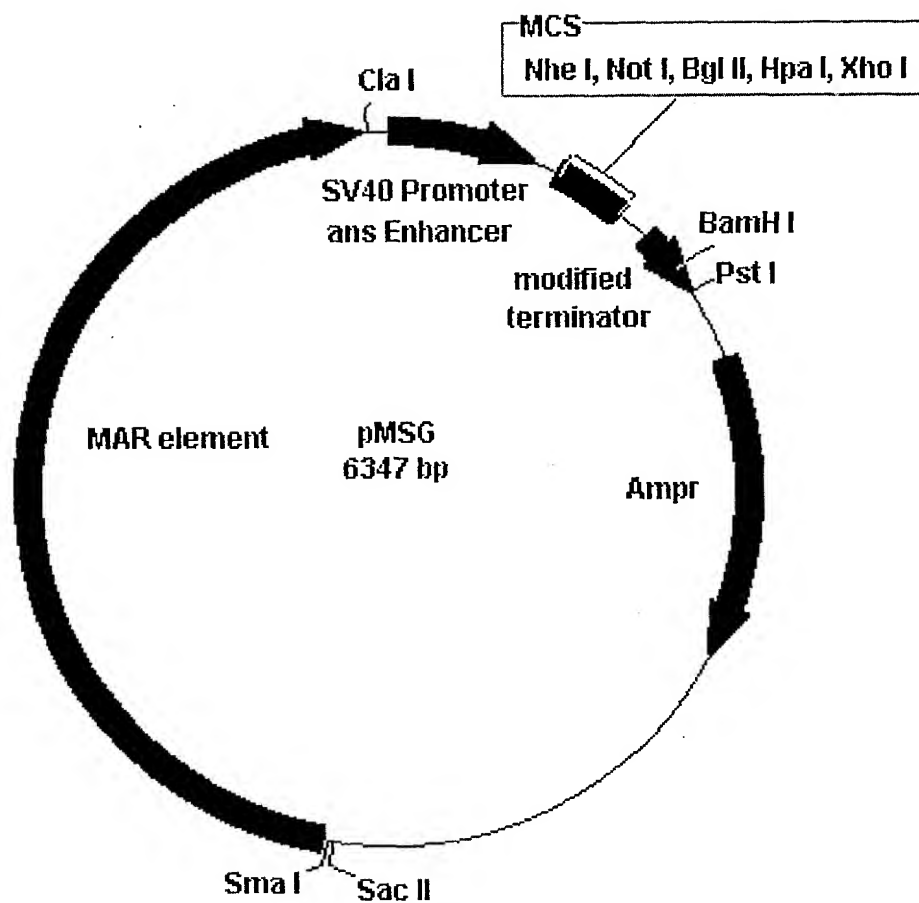
12/15

FIG. 12



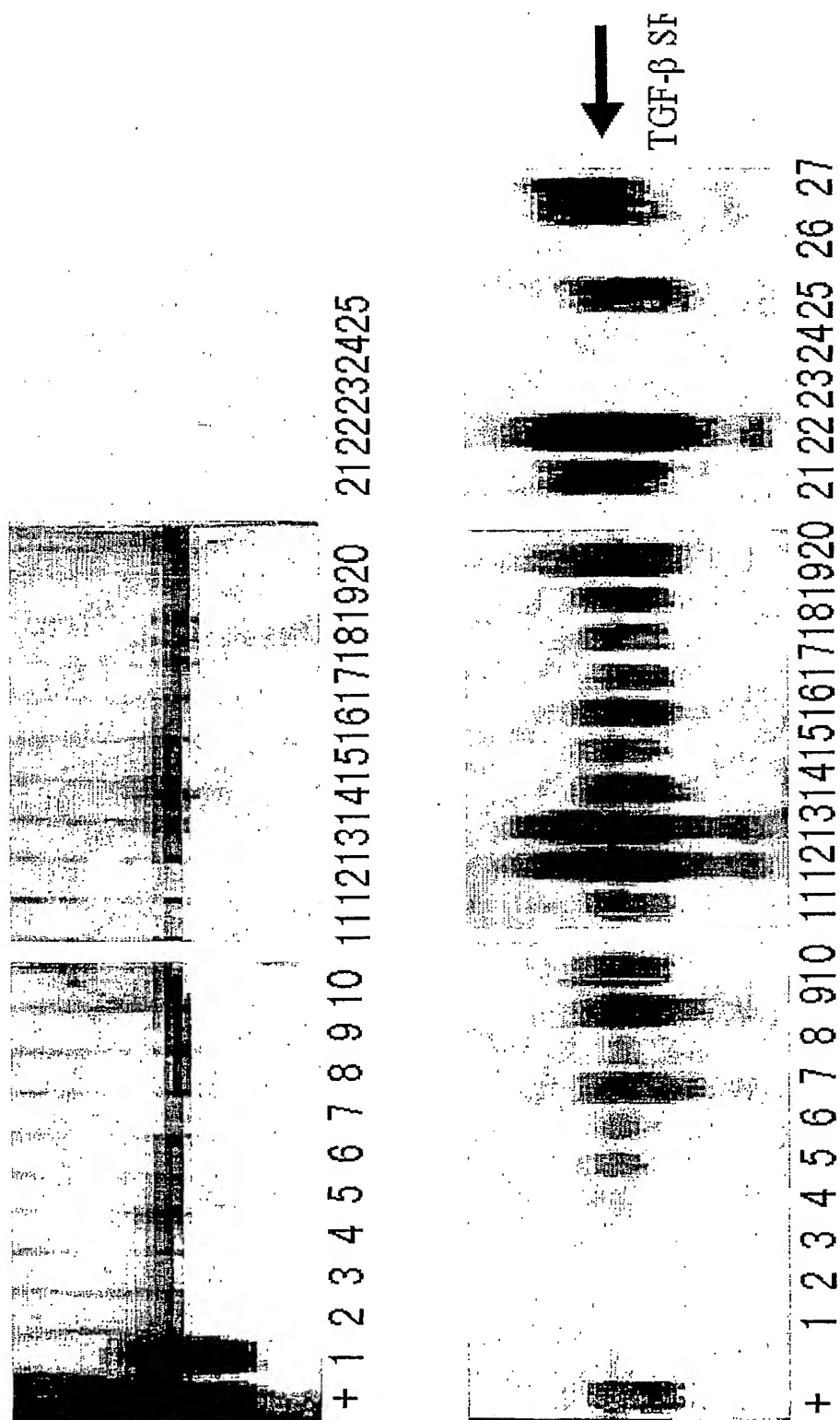
13/15

FIG. 13



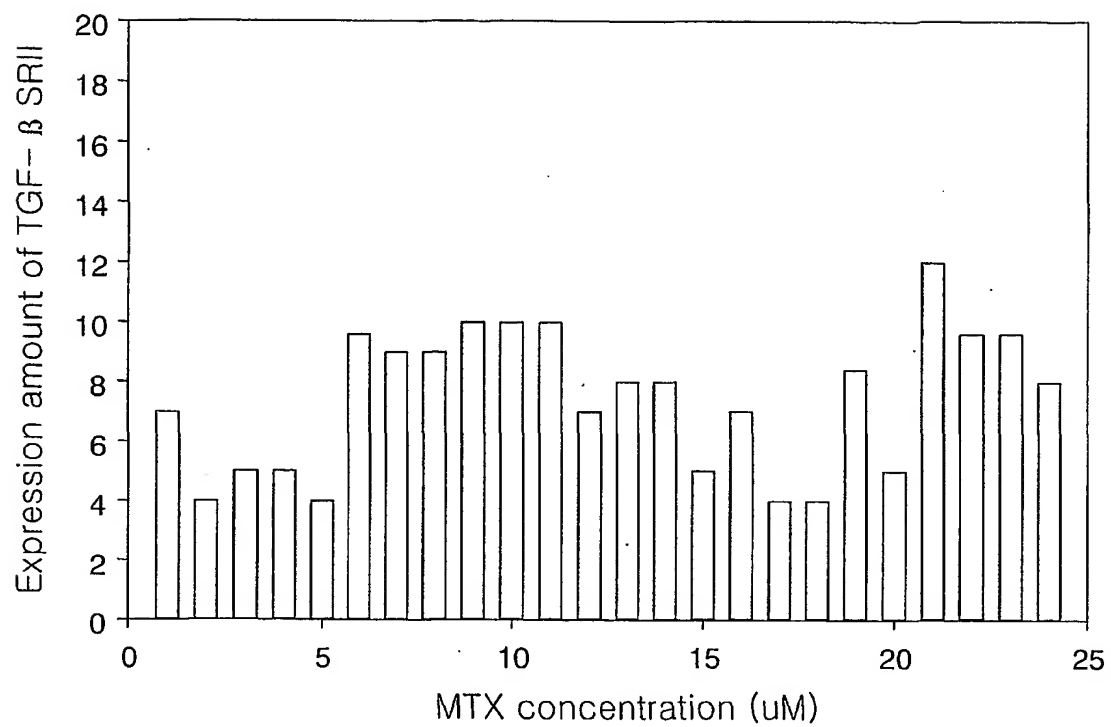
14/15

FIG.14



15/15

FIG. 15



SEQUENCE LISTING

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Pan-Gen Biotech Laboratories Inc.

<120> Expression vector for animal cell

<130> opp010629kr

<150> KR10-2000-43996

<151> 2000-7-29

<160> 24

<170> KopatentIn 1.55

<210> 1

<211> 6287

<212> DNA

<213> Artificial Sequence

<220>

<223> pMS vector sequence

<220>

<221> promoter

<222> (1)..(419)

<223> SV40virus

<220>

<221> gene

<222> (3305)..(6269)

<223> Human beta globin MAR element

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<210> 2
 <211> 435
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> gastrin fragment which is digested by Hind III restriction
 enzyme, containing poly-a signal, termination code

<220>
 <221> terminator
 <222> (256)..(435)

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```

ttgtaggcca gaggaggggg actcatctgg gctcatctgg gggatgaagt gtggcaggga 180
ctaagagctg agtgcctctt aggggcaggg accgtccccc agagccccac attgaacgag 240
aatccacagg tatgggcagg ataatatatg gtagggttca tagccagagt aacctttttt 300
tttaattttt attttatttt atttttgaga tggagtttcg ctcttgtctc ccaggctgga 360
gtgcaataat gagacctcag ctcactgcaa cctctgcctc ctaggttcaa gcgattttcc 420
tgcctcagcc tccca 435

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<210> 3
<211> 214
<212> DNA
<213> Artificial Sequence

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```

<220>
<223> SV 40 virus poly-A signal and gastrin termination site

```

```

<220>
<221> polyA_signal
<222> (1)..(135)

```

```

<220>
<221> terminator
<222> (136)..(214)

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tatcatgict ggatccagga taatatatgg tagggttcat agccagagta accttttttt 180
ttaattttta ttttttttta tttttgagct gcag 214

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<210> 4
 <211> 221
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> SV40 virus poly-A signal and reverse of gastrin poly-A signal

<220>
 <221> polyA_signal
 <222> (1)..(137)

<220>
 <221> terminator
 <222> (138)..(221)

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 aataaagcat ttttttcact gcattctagt tgtggtttgt ccaaactcat caatgtatct 120
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 <223> SV40 virus poly-A signal and gastrin poly-A signal

<220>
 <221> polyA_signal

<222> (1)..(135)

<220>

<221> terminator

<222> (136)..(217)

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<223> SV40 virus poly-A signal and minmal gastrin poly-A signal

<220>

<221> polyA_signal

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<220>

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<222> (136)..(217)

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217

<210> 7

<211> 3309

<212> DNA

<213> Artificial Sequence

<220>

<223> pSG vector sequence

<220>

<221> promoter

<222> (1)..(419)

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR01/01285

| A. CLASSIFICATION OF SUBJECT MATTER | | |
|---|--|--|
| IPC7 C12N 15/85 | | |
| According to International Patent Classification (IPC) or to both national classification and IPC | | |
| B. FIELDS SEARCHED | | |
| Minimum documentation searched (classification system followed by classification symbols) | | |
| IPC7 C12N 15/85 | | |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched | | |
| Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) | | |
| NCBI Pubmed, espacenet patent database, USPTO patent database | | |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | |
| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| X | WO0018938 (PIONEER HI BRED INT), 2000-04-06 | 1-8 |
| A | Gene 1994 Feb 25;139(2):139-45 | 1-8 |
| A | J Cell Biochem 1993 May;52(1):23-36 | 1-8 |
| A | Cell Res 1998 Sep;8(3):209-18 | 1-8 |
| A | J Cell Biochem 1999 Jul 1;74(1):38-49 | 1-8 |
| A | Nucleic Acids Res 1999 Jul 15;27(14):2924-30 | 1-8 |
| <input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex. | | |
| <p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> | | |
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| 28 DECEMBER 2001 (28.12.2001) | | 28 DECEMBER 2001 (28.12.2001) |
| Name and mailing address of the ISA/KR | | Authorized officer |
| Korean Intellectual Property Office Government Complex-Daejeon, Dunsan-dong, Seo-gu, Daejeon Metropolitan City 302-701, Republic of Korea Facsimile No. 82-42-472-7140 | | LIM, Hea Joon Telephone No. 82-42-481-5590 |

